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# Leader Peptide-Directed Processing of Labyrinthopeptin A2 Precursor Peptide by the Modifying Enzyme LabKC

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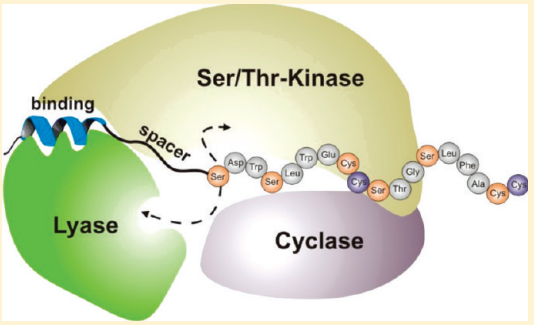
Supporting Information

**ABSTRACT:** Lantibiotics are peptide antibiotics, realizing their unique secondary structure by posttranslational modifications, the most important one being the formation of the characteristic amino acid lanthionine. Like other ribosomal peptide antibiotics, they are synthesized with an N-terminal leader peptide important for posttranslational processing by modifying enzymes; after peptide maturation, the leader peptide is proteolytically cleaved off. Numerous studies of the leader peptides of class I and II lantibiotics already showed their crucial role in recognition, self-immunity, and extracellular transport. The recently described labyrinthopeptins, members of the family of class III lantibiotics, exhibit the characteristic novel amino acid labionin, which was revealed by elucidation of the structure of labyrinthopeptin A2. The assembly of the labionin motif in the linear peptide chain is mediated by the lyase-kinase-cyclase-type enzyme LabKC through a serine side chain phosphorylation with GTP, elimination of the phosphate group, and a subsequent 2-fold Michael-type addition cyclization. In this work, we systematically investigated for the first time the importance of the leader peptide in the processing of class III lantibiotics using the example of the labyrinthopeptin A2 precursor peptide. In vitro studies with synthetic leader peptide analogues revealed that a conserved N-terminal hydrophobic patch on a putative helical structure is required for the proper peptide processing by the modifying enzyme LabKC. On the other hand, studies showed that the C-terminal part of the leader peptide serves as a spacer between the binding site and active sites for phosphorylation and elimination, thus restricting the number of hydroxy amino acid side chains that could undergo dehydration. Finally, a model for the peptide recognition and processing by the LabKC has been postulated.




Lantibiotics make up a structurally diverse group of polycyclic peptides containing thioether bridges, termed lanthionines in the amino acid side chains as a unifying structural feature (Figure 1A).[1,2] These ribosomally synthesized peptides are widely used as antibacterial agents with nisin, the most prominent representative, employed as a food preservative for decades without the appearance of any resistance.[3] The thioether cross-linkages, lanthionine and methyllanthionine, are the unifying structural motif of all lantibiotics, which are introduced subsequent to ribosomal synthesis of the linear peptide chain by various modifying enzymes. Apart from this most common posttranslational feature, more than 15 other different modifications have been reported for this group of ribosomally synthesized peptides.[4−6]

Elucidation of the structure of the recently discovered class III lantibiotic labyrinthopeptin A2 (Figure 1) from the actinomycete *Actinomadura namibiensis* revealed the triamino triacid labionin as a new posttranslational modification.[7−9] In this motif, the lanthionine rings are extended by an additional N-terminal ring, forming a carbacyclic side chain linkage and a quaternary $\alpha$C atom. Sequencing of the labyrinthopeptin gene cluster revealed two structural genes (*lab*A1 and *lab*A2) for precursor peptides of labyrinthopeptin A2 and A1/A3, two sequences encoding putative ABC transporters (*lab*T1 and *lab*T2), and one gene for the labyrinthopeptin-modifying enzyme LabKC (*lab*KC).[7] The sequence of LabKC consists of a highly conserved central domain homologous to human like Ser/Thr protein kinases from *Mycobacterium tuberculosis* with a conserved active site and a nucleotide binding motif.[10,11] A C-terminal section shows a low degree of homology to lanthionine cyclase enzymes; however, crucial residues for zinc binding involved in the activation of the cysteine side chain identified for nisin cyclase are missing in the sequence.[12] The N-terminal domain is analogous to phospholyase enzymes catalyzing the elimination reaction of the phosphorylated serines/threonines as recently proposed for the modifying enzyme VenL from *Streptomyces venezuelae*.[13−17]

A general mechanism for the synthesis of lanthionines from lantibiotic precursor peptides consists of the dehydration of serine or threonine residues resulting in 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) formation and the subsequent Michael-type addition of an activated cysteine side chain that completes formation of the thioether bridge.[1] Recently, we succeeded in establishing an in vitro enzyme assay for the biosynthesis of labyrinthopeptin A2 attached to the

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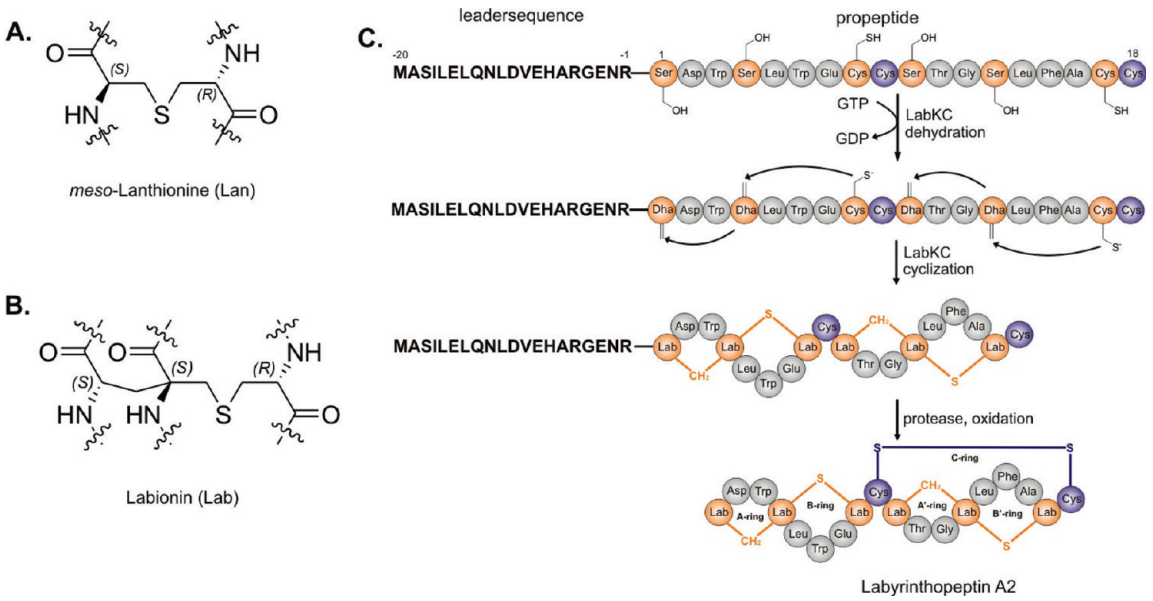


**Figure 1.** Structures of (A) lanthionine (Lan), characteristic of lantibiotics, and (B) labionin (Lab), characteristic of the class III lantibiotic labyrinthopeptin. (C) Proposed scheme of the basic principles of labyrinthopeptin A2 biosynthesis. After ribosomal synthesis, the precursor peptide of labyrinthopeptin A2 is processed by the three-domain enzyme LabKC. Phosphorylation of serine residues and subsequent elimination result in the formation of 2,3-didehydroalanine (Dha). Labionin formation would be accomplished by a putative 2-fold Michael-type addition initiated by nucleophilic attack of the activated cysteine side chain.

leader peptide.[18] We were able to show the successful conversion of a synthetic linear labyrinthopeptin A2 precursor peptide (LabA2) into the fully dehydrated and cyclized peptide by incubation with the recombinantly expressed modifying enzyme $LabKC_{His6}$. Surprisingly, GTP was the cosubstrate for the phosphorylation step rather than ATP as previously reported, e.g., lacticin 481 and haloduracin lantibiotic synthetases.[19,20] Formation of the labionin motif was proposed by the dehydration of the serine residues in the conserved Ser-$(Xxx)_2$-Ser-$(Xxx)_{3/5}$-Cys motif and subsequent ring formation by a 2-fold Michael-type addition, which is induced by the nucleophilic attack of a C-terminal cysteine side chain.[18]

A common feature of ribosomally encoded peptide antibiotics is the synthesis as a precursor peptide, with a C-terminal core peptide, which undergoes posttranslational modifications, and an N-terminal part termed the leader sequence.[1,6] Various functions have previously been suggested for leader peptides of lantibiotics as well as of other ribosomal peptide antibiotics, e.g., microcins, thiopeptides, or cyanobactins.[21] Whereas leader peptides are described as a self-immunity mechanism for the producer strain, they also serve as a general recognition motif for the processing enzymes and transport systems.[21,22] After posttranslational modification is accomplished, the leader peptide is usually cleaved off from the mature peptide by proteases before, during, or after the export from the cytoplasm into the extracellular environment. The importance of the leader peptide for successful enzymatic processing of lantibiotics by the modifying machinery has already been shown for class I and II peptides.[23−29] In this study, we present insights into the requirements for the leader peptide-dependent processing of class III lantibiotics, with the example of the processing of synthetic labyrinthopeptin A2 leader peptide analogues by the modifying enzyme LabKC. The identification of a minimal motif required for processing was accomplished by testing a series of truncated leader peptides. Additionally, three crucial conserved hydrophobic residues were identified by single-amino acid exchanges in the leader peptide.

## MATERIALS AND METHODS

**Materials.** *Cloning of LabKC.* The *labKC* gene was amplified by polymerase chain reaction (PCR) from a cosmid[7] by using custom-synthesized primers (LabKC_*Eco*RI fw, 5′-TACGAATTCATGGATCTGCGGTACCACGC-3′; LabKC_XhoI rv, 5′-ATATCTC-GAGCCTCCTCCCCCGGGGTCGCGT-3′). The resulting PCR product was gel-purified with the QiaexII DNA extraction kit (Qiagen, Hilden, Germany) and digested with EcoRI and XhoI before being ligated into vector pET24a(+) (Merck, Darmstadt, Germany), encoding a C-terminal hexahistidine tag. The sequence of the pET24-LabKC construct was verified by DNA sequencing. No mutations or frameshifts were detected.

*Protein Expression and Purification.* *Escherichia coli* BL21-(DE3) cells were transformed with the pET24-*labKC* construct. For overexpression, cells were grown in LB medium with 50 $\mu$g/mL kanamycin at 37 °C and induced with 0.15 mM isopropyl $\beta$-D-thiogalactoside (IPTG) when the culture reached an $OD_{600}$ of 0.6−0.8. After induction, the culture was grown for an additional 48 h at 14 °C, and subsequently, cells were harvested by centrifugation. Cells were resuspended in lysis buffer [50 mM Tris-HCl, 1 M NaCl, 20 mM imidazole, 1 mM DTT, and 0.1 mM PMSF (pH 8.0)], disrupted with a cell homogenizer Emulsiflex (Avestin, Inc., Ottawa, ON) for two runs, and centrifuged for 20 min at 20000*g* to remove cell debris. All following purification steps were performed at 4 °C. The $LabKC_{His6}$ protein was purified by nickel affinity chromatography using His-Buster Affinity Gel (Amocol Bioprocedures Ltd., Teltow, Germany). After binding, the resin was washed three times with 50 mL of 50 mM Tris-HCl, 1 M NaCl, 20 mM imidazole, and 1 mM DTT (pH 8.0), and bound protein was subsequently eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, and 1 mM DTT (pH 8.0).


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Table 1. Summary of Laba2 Peptide Variations and Truncated Leader Peptides Used to Study the Dehydration Activity by Labkc. Highlighted Amino Acids Indicate Conserved Ser Residues Involved in Phosphorylation and Dehydration (Yellow) and Replacement or Insertion of Amino Acids in the Leader Sequence (Green)[a]

| Entry | Peptide Sequence: Leader peptide -20 … -1 +1 … +18 Core peptide | Expected dehydrations | Observed number of dehydrations |
|---|---|---|---|
| (1) LabA2 | MASILELQNLDVEHARGENR-SDWSLWECCSTGSLFACC | 4 | 4 |
| (2) LabA2* | MASILELQNLDVEHARGENR-SDWSLWECASTGSLFACA | 4 | 4 |
| (3) LabA2$_{-20\text{-}1}$CONH$_2$ | MASILELQNLDVEHARGENR-CONH$_2$ | 0 | 0 |
| (4) Ac-LabA2*$_{1\text{-}18}$ | Ac-SDWSLWECASTGSLFACA | 4 | 0 |
| (5) LabA2$^{**}$ | MASILELQNLDVEHARGENR-SDWSLWECA | 2 | 2 |
| | *N-terminal truncations of leader peptide* | | |
| (6) LabA2*$_{\Delta(-20\ -14)}$ | LQNLDVEHARGENR-SDWSLWECASTGSLFACA | 4 | 0 |
| (7) LabA2*$_{\Delta(-20\ -7)}$ | HARGENR-SDWSLWECASTGSLFACA | 4 | 0 |
| (8) LabA2**$_{\Delta(-20\ -19)}$ | SILELQNLDVEHARGENR-SDWSLWECA | 2 | 2 |
| (9) LabA2**$_{\Delta(-20\ -18)}$ | ILELQNLDVEHARGENR-SDWSLWECA | 2 | 2 |
| (10) LabA2**$_{\Delta(-20\ -17)}$ | LELQNLDVEHARGENR-SDWSLWECA | 2 | 0 |
| (11) LabA2**$_{\Delta(-20\ -16)}$ | ELQNLDVEHARGENR-SDWSLWECA | 2 | 0 |
| | *C-terminal truncations of leader peptide* | | |
| (12) LabA2*$_{\Delta(-8\ -2)}$ | MASILELQNLDV-------R-SDWSLWECASTGSLFACA | 4 | 2 |
| (13) LabA2*$_{\Delta(-12\ -2)}$ | MASILELQ----------R-SDWSLWECASTGSLFACA | 4 | 1 |
| (14) LabA2*$_{\Delta(-14\ -2)}$ | MASILE----------R-SDWSLWECASTGSLFACA | 4 | 0 |
| (15) LabA2*$_{shift}$ | MASDVEHAILELQNLRGENR-SDWSLWECASTGSLFACA | 4 | 2 |
| (16) LabA2**_ex. | MASILELQNLDVEHARDLQAGENR-SDWSLWECA | 2 | 2 |
| (17) SapB$_{-20\ -1}$_LabA2$_{1_9}$ | MNLFDLQSMETPKEEAMQDV-SDWSLWECA | 2 | 2 |

[a]Highlighted amino acids indicate conserved Ser residues involved in phosphorylation and dehydration and replacement or insertion of amino acids in the leader sequence.

Fractions containing the eluted protein were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for purity and were further purified by size exclusion chromatography using a Superdex 200 10/300 GL column coupled to an ÄKTA Purifier 10 system (GE-Healthcare, Uppsala, Sweden). Protein concentrations were determined spectrophotometrically resulting in 0.5 mg of protein/L of culture. The purified LabKC-His$_6$ enzyme was directly used for activity assays or alternatively stored at −80 °C.

*Peptide Synthesis and Purification.* All peptide substrates were synthesized by automated solid-phase peptide synthesis using standard protocols for Fmoc/*t*Bu strategy (see the Supporting Information). Subsequent to ether precipitation and lyophilization, crude peptides were further purified by size exclusion chromatography and preparative reverse-phase high-performance liquid chromatography (RP-HPLC) (see the Supporting Information).

*LabKC Activity Assays.* For the LabKC functionality assay, the labyrinthopeptin LabA2 precursor peptides were redissolved in 50 mM Tris buffer (pH 8.0) and the concentration was determined spectrophotometrically. The assay mixture with a total volume of 50 $\mu$L consisted of 10 $\mu$M LabA2 precursor peptide [including variants (see Table 1)], 20 mM TES (pH 7.0), 2 mM GTP, 10 mM $MgCl_2$, 1 mM DTT, and 0.7 $\mu$M LabKC$_{His6}$ with an enzyme:substrate ratio of ∼1:14. For comparison of results, incubation of control samples without enzyme was used. After incubation for 2 h at 28 °C, the assay mixture was quenched with a methanol/water mixture (1:1) and centrifuged to remove precipitated protein. Precipitation of peptide substrates was not observed at this methanol

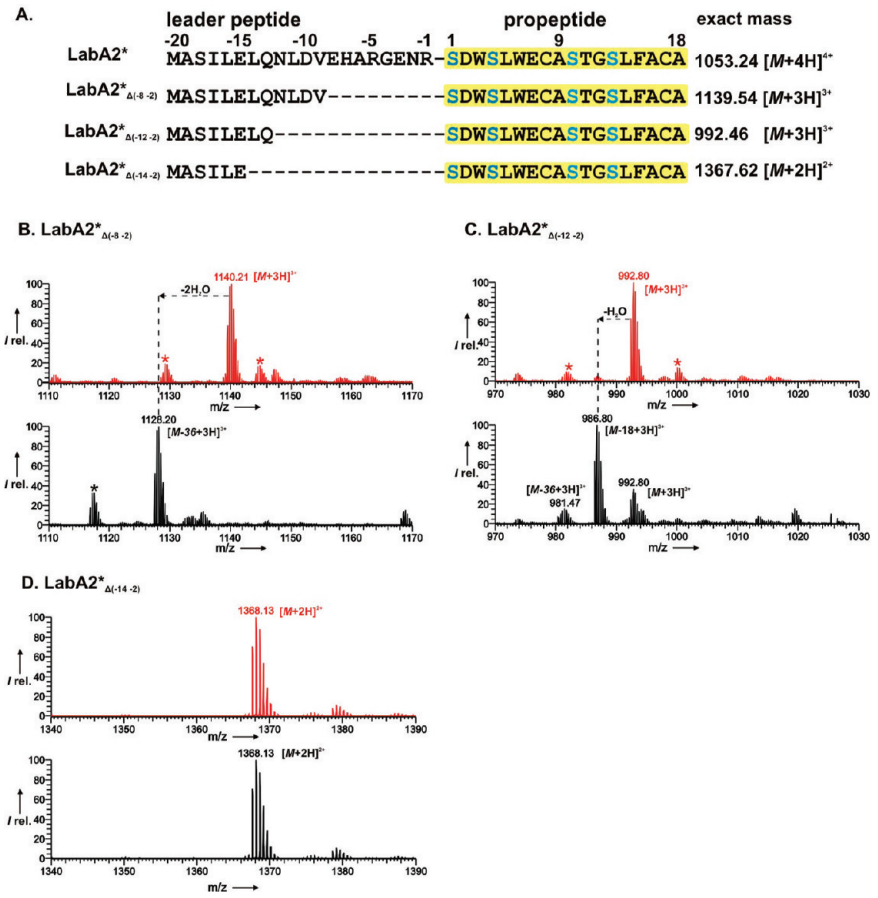


**Figure 2.** (A) Sequences of C-terminally truncated leader peptides tested for conversion with $LabKC_{His6}$. (B and C) LC−MS spectra (red, unmodified peptide; black, peptide incubated with LabKC) of assay peptides showing $[M + 3H]^{3+}$ ions for peptides $LabA2^*_{\Delta(-8-2)}$ (B) and $LabA2^*_{\Delta(-12-2)}$ (C). No conversion was observed for peptide $LabA2^*_{\Delta(-14-2)}$ ($[M + 2H]^{2+}$) after incubation with $LabKC_{His6}$ (D). Asterisks indicate side products of peptide synthesis.

concentration. For liquid chromatography−electrospray ionization tandem mass spectroscopy (LC−ESI-MS/MS) experiments, the assay mixture after incubation with $LabKC_{His6}$ was directly treated with 1−2 μg of modified trypsin (New England Biolabs, Frankfurt, Germany) and incubated for 2 h at 37 °C. After incubation, the digest was quenched with a methanol/water mixture (1:1), centrifuged, and analyzed by LC−ESI-MS/MS.

*LC−MS and LC−MS/MS Conditions.* HR-ESI-MS spectra were recorded on an Exactive-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany). For chromatographic separation of assay products, a C18 Grom-Sil 120 ODS column (3 μm, 100 mm × 2 mm) (Alltech Grom GmbH, Rottenburg-Hailfingen, Germany) with a linear solvent gradient from 20 to 80% solvent B (solvent A, water with 0.1% HCOOH; solvent B, ACN with 0.1% HCOOH) over 10 min was used. Additional LC−ESI-MS experiments were performed on a qTof 2 hybrid quadrupole time-of-flight ESI mass spectrometer (Micromass/Waters) coupled to an Agilent 1200 HPLC system (Agilent, Waldbronn, Germany) using the same gradient and column mentioned above. LC−ESI-MS/MS experiments were conducted using a LTQ-Orbitrap XL (Thermo Scientific) coupled to an Agilent 1260 HPLC system (Agilent Technologies). For chromatographic separation, a Vydac 218M C18 5 μm, 150 mm × 2.1 mm column (Grace) with a linear solvent gradient from 5 to 100% solvent B over 20 min was used. Solvent A was water (0.1% HCOOH). The MS/MS spectra were recorded in FTMS mode with fragmentation in the HCD cell (a normalized collision energy of 18−25% was used).

*Circular Dichroism (CD) Measurements.* CD spectra were recorded with a J715 CD spectrometer (JASCO, Gross-Umstadt, Germany) at 25 °C using a SUPRASIL quartz cuvette, with a path length of 1 mm (Hellma, Müllheim, Germany). The ellipticity (millidegrees) of peptide solutions in water and different concentrations of trifluoroethanol was measured over a wavelength range of 185−260 nm using five scans. Spectra were smoothed (Savitsky−Golay smoothing, smoothing window 15 point) and converted into molar ellipticity.

*Secondary Structure Predictions.* Secondary structure prediction of the LabA2 leader peptide was performed using the PHD algorithm (http://www.ebi.ac.uk/~rost/predictprotein) and JPred (http://www.compbio.dundee.ac.uk/www-jpred/).

## RESULTS

**Disulfide-Forming Cysteines Are Not Required for LabKC Peptide Recognition and Dehydration.** Unlike other class III lantibiotics, the structure peptide of labyrinthopeptin A2 contains an unusually high number of cysteines.[1,7] From the double Cys motifs C-8/C-9 and C-17/C-18, only C-8 and C-17 are involved in labionin formation, whereas C-9 and


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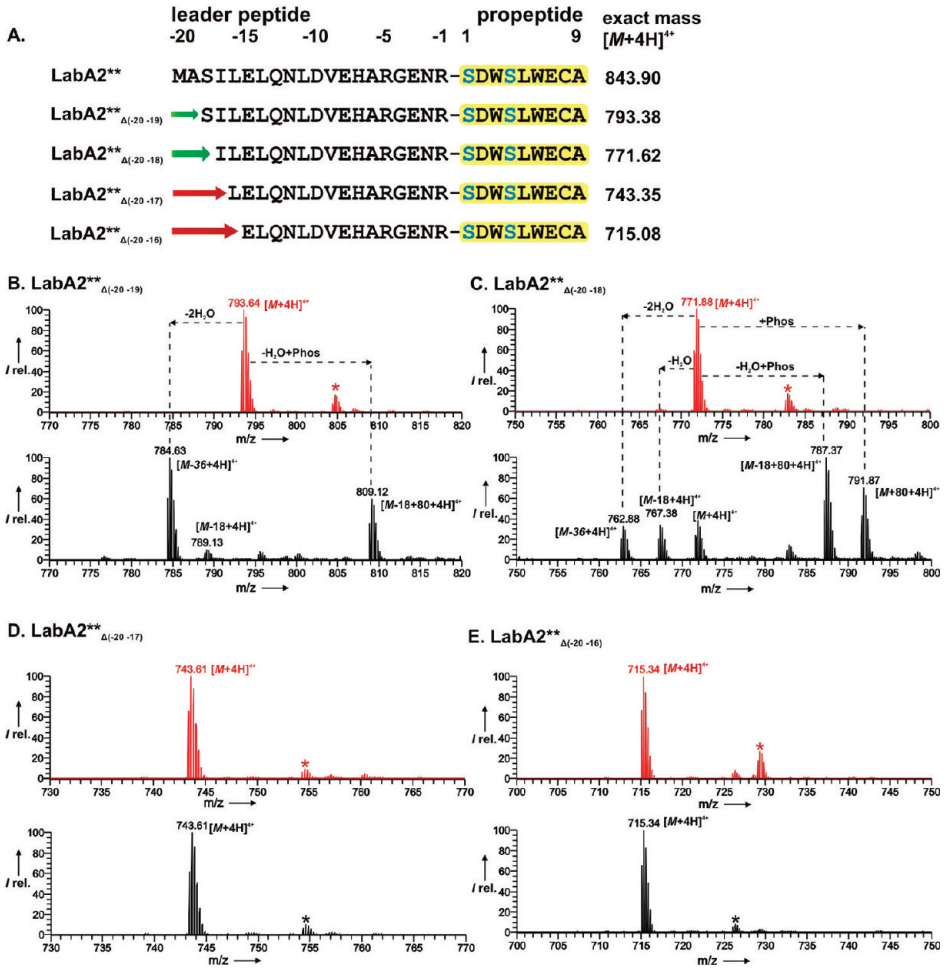


**Figure 3.** (A) Sequences of N-terminally truncated leader peptides tested for conversion with $LabKC_{His6}$. (B–E) LC–MS spectra (red, unmodified peptide; black, peptide incubated with LabKC) of assay products showing $[M + 4H]^{4+}$ ions. Whereas $LabA2^{**}{}_{\Delta(-20-19)}$ (B) and $LabA2^{**}{}_{\Delta(-20-18)}$ (C) were still dehydrated by $LabKC_{His6}$, no conversion was observed for N-terminally truncated peptides $LabA2^{**}{}_{\Delta(-20-17)}$ (D) and $LabA2^{**}{}_{\Delta(-20-16)}$ (E). Asterisks indicate side products of peptide synthesis.

C-18 form the disulfide bond of ring C (Figure 1). To assess the influence of C-9 and C-18 contributing to ring C of labyrinthopeptins on the processing activity of LabKC, we replaced C-9 and C-18 with Ala [$LabA2_{C\text{-}9A,C\text{-}18A}$ (Table 1, entry 2)]. The LC–MS analysis of $LabA2_{C\text{-}9A,C\text{-}18A}$ incubated with recombinantly expressed and purified $LabKC_{His6}$ showed that full phosphorylation and dehydration were retained compared to those of LabA2 (see Figure S1 of the Supporting Information). Because considerable difficulties in the synthesis of precursor peptide analogues were pinpointed to the synthesis of the double-Cys motif in the structure of LabA2, mostly C-9A/C-18A peptide substrates (termed LabA2*) with these exchanges were used in subsequent experiments.

**The Leader Peptide Is Essential for the Dehydration Activity of LabKC.** With regard to conversions of precursor peptides by LabKC, the possibility that the absence of the leader peptide still allows recognition and dehydration of the structure peptide by LabKC could not be ruled out. Therefore, we tested a LabA2 core peptide ($Ac\text{-}LabA2^*{}_{1-18}$) lacking the N-terminal leader sequence as a substrate for $LabKC_{His6}$ (Table 1, entry 4). The N-terminus was acetylated to prevent undesired ionic interactions, thus reflecting an appropriate peptide context. Incubation with $LabKC_{His6}$ and the cosubstrate GTP revealed in subsequent LC–MS analyses that this peptide showed no conversion; i.e., it lacked phosphorylations or dehydrations found for incubations with the full-length peptide LabA2* (Figure S2 of the Supporting Information). Also, assay conditions providing additionally a C-terminally amidated leader peptide ($LabA2_{-20-1}CONH_2$) in trans to the reaction were devoid of modifications by $LabKC_{His6}$ (Figure S3 of the Supporting Information). Hence, these experiments strongly suggest that the leader sequence is mandatory for the enzymatic processing of the labyrinthopeptin A2 core peptide by the modifying enzyme LabKC.

**Influence of Leader Peptide Truncations on LabA2 Processing.** To further elucidate the motif of the leader peptide responsible for recognition and binding, peptides with truncated leader sequences from both the N- and C-termini were tested. Enzymatic activity was scored by the number of phosphorylations and dehydrations observed, compared to the number of phosphorylations and dehydrations observed for the LabA2* peptide under the same experimental conditions. For each in vitro experiment, equal concentrations of peptide substrate and $LabKC_{His6}$ were used to maintain the reproducibility and comparability of modification efficiency. Two N-terminally truncated leader peptide variants with deletions of six amino acids [$LabA2^*{}_{\Delta(-20-14)}$] and 13 amino acids [$LabA2^*{}_{\Delta(-20-7)}$] (Table 1) were not modified by


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$LabKC_{His6}$ as shown by LC−MS analysis (Figure S4 of the Supporting Information). In contrast, deletions at the C-terminus of the leader peptide with removal of seven [$LabA2^*_{\Delta(-8-2)}$] and 11 amino acids [$LabA2^*_{\Delta(-12-2)}$] were still recognized and modified in the $LabKC_{His6}$ enzyme assay. However, incubation resulted in incomplete dehydration of only the four serines in the core peptide. Hence, the major assay products were peptides with two dehydrations [$LabA2^*_{\Delta(-8-2)}$] (Figure 2B) and $LabA2^*_{\Delta(-12-2)}$ with only one dehydration (Figure 2C). A third C-terminally truncated leader peptide containing only the first six amino acids from the N-terminus [$LabA2^*_{\Delta(-14-2)}$] was not modified (Figure 2D). From these initial experiments, we concluded that shortening of the C-terminus of the leader peptide to only six amino acids results in abrogation of peptide modification activity by LabKC. For subsequent studies, we decided to preferably use LabA2* peptides in a further truncated core peptide version, termed LabA2** (Table 1), as these peptides were fully dehydrated by LabKC and could be more easily and more quickly accessed by chemical synthesis (see Figure S5 of the Supporting Information).

To further elucidate the essential motif of the LabA2 precursor peptide for LabKC processing activity, we continued to test peptides containing stepwise single-amino acid deletions from the N-terminus. Results in Figure 3 show that whereas peptide $LabA2^{**}_{\Delta(-20-19)}$ was still fully dehydrated, truncation of three N-terminal amino acids [$LabA2^{**}_{\Delta(-20-18)}$] already had a significant influence on peptide processing as accumulation of phosphorylated intermediates showed. The N-terminal deletion of further amino acids, e.g., $LabA2^{**}_{\Delta(-20-17)}$ and $LabA2^{**}_{\Delta(-20-16)}$, fully abolished dehydration by LabKC. Consequently, truncation of the leader peptide from the N-terminus is tolerated until the critical position of Ile-17, indicating that this residue plays a crucial role in enzyme recognition.

To address the positions of Ser residues that were dehydrated in the C-terminal leader truncated peptides $LabA2^*_{\Delta(-8-2)}$ and $LabA2^*_{\Delta(-12-2)}$ (Table 1, entries 12 and 13), we performed LC−MS/MS experiments with the 2-fold and singly dehydrated peptides after removal of the leader peptide by tryptic digestion. The data of the 2-fold dehydrated peptide clearly show that in the case of $LabA2^*_{\Delta(-8-2)}$ Ser10 and Ser13 were dehydrated by $LabKC_{His6}$ whereas Ser1 and Ser4 escaped modification (Figure S6 of the Supporting Information). In addition, the data suggest cyclization of the C-terminal A′B′ ring of the peptide as no fragmentation was observed in this region. Likewise, fragmentation results for the singly dehydrated $LabA2^*_{\Delta(-12-2)}$ peptide show that dehydration occurred on the C-terminally located serine residue (Figure S7 of the Supporting Information).

Altogether, these results suggest that the LabA2 precursor peptide is recognized and/or properly positioned by LabKC via a specific motif in the N-terminal part of the leader peptide as N-terminally truncated peptides were not modified. Secondly, the C-terminal part of the leader would act as a spacer between the recognition site of the leader and the active sites for phosphorylation and elimination. This spacer distance constitutes a crucial and limiting factor for Ser residues destined for dehydration. We further confirmed this assumption with a peptide bearing a Glu → Ser exchange ($LabA2^{**}_{E\text{-}3S}$) within the leader peptide. After incubation with LabKC, LC−MS and LC−MS/MS analysis showed that the two observed dehydrations occurred only in the core peptide region and that Ser-3 in the leader peptide was not affected (Figure S8 of the Supporting Information).

On the other hand, elongation of the leader peptide in this putative spacer region by a randomly chosen tetrapeptide [LabA2**_ex. (Table 1, entry 16)] had no influence on processing. LC−MS spectra of the assay mixture after incubation with $LabKC_{His6}$ show clearly the fully 2-fold dehydrated peptide as the major product (Figure S9 of the Supporting Information). These results confirm the hypothesis that the C-terminal part has no function in recognition but rather acts as a linker region.

**Processing by LabKC Is Facilitated by a Characteristic Peptide Motif in the N-Terminal Part of the Leader Peptide.** Protein-based multiple-sequence alignments of class III lantibiotics (LabA1, LabA2, SapB, and analogous peptides from *Streptomyces avermitilis*, *Streptomyces griseus*, and *Streptomyces erythraea*) revealed a highly conserved region in the N-terminal part of the leader peptide with mainly hydrophobic amino acids and the consensus motif B-A-C-Leu-Gln (B is Ile, Leu, or Val; A is Phe or Leu; C is Glu or Asp).[30−35] Additionally, secondary structure prediction tools suggest the likely formation of an α-helix in this part of the leader peptide (Figure 4).[36,37] To further prove that the conserved N-terminal

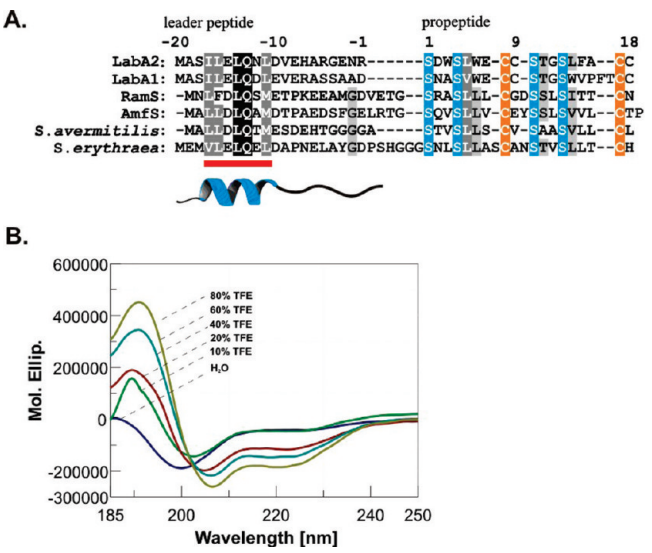


**Figure 4.** (A) Multiple-sequence alignment of class III lantibiotic precursor peptides from *A. namibiensis* (LabA1 and LabA2), *S. griseus* (Griseopeptin), *S. avermitilis* (Avermipeptin), and *Saccharopolyspora erythraea* (Erythreopeptin) and the SapB morphogen from *Streptomyces coelicolor*. The highly conserved part is indicated by a red bar together with the proposed helix-forming region (according to the secondary structure prediction). Ser and Cys residues that are involved in labionin formation are highlighted (light blue and brown). (B) Circular dichroism spectra of the amidated LabA2 leader peptide in water and various concentrations of TFE.

part of the leader peptides described above is of more general significance for the processing, we synthesized a chimeric peptide containing the first 20 amino acids of the SapB leader peptide and the truncated core peptide of LabA2 [$SapB_{-20-1}\_LabA2_{1-9}$ (Table 1, entry 17)]. This peptide was fully dehydrated by LabKC, indicating that the leader sequence of SapB is also recognized by LabKC (Figure S10 of the Supporting Information).


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**Characterization of the LabKC Recognition Motif of the Leader Peptide by Amino Acid Substitutions.** For deeper characterization of the crucial sites of leader peptide recognition and their involvement in processing by LabKC, we performed single-amino acid substitutions in the N-terminal part of the leader peptide (Figure 4). Initially, we started with an Ala scan of individual residues in the conserved ILELQ motif. Peptides with exchanges of hydrophobic residues I-17A, L-16A, and L-14A were still processed but with an efficiency lower than that of the wild-type derived peptide LabA2** (Figure S11 and Table S1 of the Supporting Information). On the other hand, exchanges of single amino acids with Ala residues C-terminally from the conserved ILELQ motif of the leader peptide had no significant effect on enzymatic activity (Figures S11 and S12 and Table S1 of the Supporting Information). Likewise, exchanging the acidic Glu in the leader sequence of peptide LabA2**$_{E\text{-}15A}$ or Gln in peptide LabA2**$_{Q\text{-}13A}$ with Ala had no effect, as the expected 2-fold dehydrated product was obtained by conversion with LabKC (Figure S11 and Table S1 of the Supporting Information). Therefore, interactions with LabKC via the conserved acidic side chain of E-15 were excluded. This Ala scan series was further complemented by an Asp scan of leader peptide positions considered crucial for leader peptide recognition (Figure 5). By exchanging I-17, L-16, and L-14 with the acidic

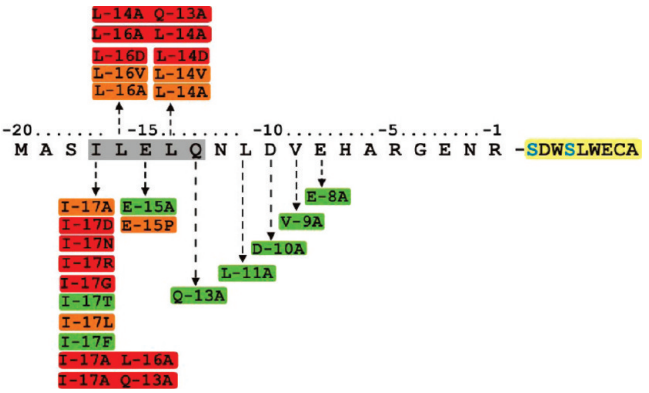


**Figure 5.** Overview of various amino acid exchanges performed in the leader peptide. Peptides are labeled according to the conversion efficiency after incubation with LabKC$_{His6}$ (green, complete or nearly complete conversion to a 2-fold dehydrated peptide as the major product; orange, limited conversion with accumulation of intermediates; red, no or negligible conversion). LC–MS spectra and detailed information about the conversion efficiency and product distribution patterns are given in the Supporting Information.

amino acid aspartate (peptides LabA2**$_{I\text{-}17D}$, LabA2**$_{L\text{-}16D}$, and LabA2**$_{I\text{-}14D}$, respextively), the conversion efficiency was almost completely abolished. Only for LabA2**$_{L\text{-}16D}$ could small amounts of singly dehydrated product be observed by LC–MS analysis (Figure S13 and Table S1 of the Supporting Information). Further exchanges of the side chain functionality of Ile-17 revealed that LabA2**$_{I\text{-}17N}$ and LabA2**$_{I\text{-}17R}$ were virtually unmodified, in contrast to substitution with Thr (LabA2**$_{I\text{-}17T}$) and Phe (LabA2**$_{I\text{-}17F}$) (Figures S13–S15 and Table S1 of the Supporting Information). To improve our understanding of the importance of the ILELQ motif, we performed double Ala substitutions of Ile, Leu, and Gln. All four tested double mutants displayed either extremely suppressed processing (LabA2**$_{I\text{-}17A,Q\text{-}13A}$ and LabA2**$_{L\text{-}14A,Q\text{-}13A}$) or no processing at all (LabA2**$_{I\text{-}17A,L\text{-}16A}$ and LabA2**$_{L\text{-}16A,L\text{-}14A}$) (Figure S11 of the Supporting Information and Figure 5), indicating that double substitutions in this crucial motif are not tolerated by the enzyme LabKC.

In a further experiment, we synthesized a full-length LabA2 precursor peptide in which an ILELQNL motif (conserved residues ILELQ) of the leader peptide was shifted to the center of the leader peptide (LabA2*$_{shift}$). After incubation with LabKC, we found two of the expected four dehydrations as the major product and small amounts of singly and triply dehydrated peptide (Figure S16 of the Supporting Information). MS/MS experiments with the doubly dehydrated product localized Dha in positions 4 and 13 of the core peptide (Figure S16 of the Supporting Information). Hence, it is possible to shift the conserved recognition motif away from the N-terminus.

**Kinetic Analysis of Processing of the LabA2 Leader Peptide Mutants.** In addition to the end point analysis, we performed simplified kinetic investigations of selected precursor peptide mutants. Reaction mixtures were set up as described previously and upon quenching of reactions (after 1.0, 2.5, and 6.0 min) analyzed by LC–MS (see Figure S19 of the Supporting Information). The progress curves were plotted on the basis of the depletion of substrate followed by extracted ion chromatograms (XIC). Peptide LabA2**$_{I\text{-}17D}$ showed no conversion within this time range. Alanine mutants of conserved Leu (LabA2**$_{L\text{-}14A}$ and LabA2**$_{L\text{-}16A}$) residues display significantly lowered conversion rates compared to that of the wild-type-derived peptide LabA2**. Interestingly, the reaction rate of the chimeric SapB–LabA2 peptide (SapB$_{-20-1}$_LabA2$_{1-9}$) is higher than that of the alanine mutants, providing additional evidence of the importance of the ILELQ motif.

**Structural Analysis of the LabA2 Leader Peptide.** To further determine if the side chains in the conserved motif are arranged by an $\alpha$-helix as indicated by the secondary structure prediction, we tested peptides with single substitutions with residues known for inducing conformational changes, i.e., Pro and Gly (LabA2**$_{E\text{-}15P}$ and LabA2**$_{I\text{-}17G}$).[38] These peptides could still be processed by LabKC, however, with a lower efficiency that resulted in only one of two dehydrations of serines (Figure S17 and Table S1 of the Supporting Information).

To assess secondary structure formation of the leader peptide, we performed CD measurements with the amidated leader peptide (LabA2$_{-20-1}$CONH$_2$). Determination of structural features by means of CD spectroscopy has already been used for lantibiotics as well as microcins.[39,40] Whereas measurements in water gave no specific signal, addition of increasing concentrations of trifluoroethanol (TFE) enhanced the helix formation with characteristic minima ($\lambda$) at 207 and 220 nm (Figure 4B). Helix formation in peptides by trifluoroethanol was previously described.[39] This observation confirms that the leader peptide of LabA2 can principally adopt a helical structure. With regard to these results, we favor the idea of peptide recognition by a hydrophobic patch consisting of the side chains of the conserved ILELQ motif that are arranged by $\alpha$-helical structure in this region.

## DISCUSSION

Ribosomally synthesized peptide antibiotics undergoing post-translational modifications contain amino-terminal leader

peptides. These peptides are removed in a final step of peptide maturation and commonly do not undergo any other modifications. The structure, function, and importance of leader peptides for recognition by modifying enzymes, transport, and immunity systems have already been described for multiple examples.[21] For lantibiotics, extensive investigations of these aspects have been performed for class I and II lantibiotics both in vitro and in vivo.[23−26]

In this study, we examined for the first time the role of the leader peptide of class III lantibiotics. Interestingly, modifying enzymes of this lantibiotic class display a unique structural architecture. The recently described LabKC enzyme is composed of three well-defined domains: lyase, Ser/Thr-kinase, and lanthionine cyclase,[10−12,16] which is also supported by homology searches of the NCBI Protein Database. In contrast, modifying enzymes from class I and II do not display such well-defined domain architecture (based on sequence analysis). Thus, we believe that the recognition or requirements for a leader peptide might be different for class III lantibiotics compared to the already well characterized systems of classes I and II. With respect to this, we initiated a systematic mapping of the recognition site of the 20mer LabA2 leader peptide for the recognition by the processing enzyme LabKC in vitro. In the described enzyme assays, peptide variants obtained from solid-phase peptide synthesis undisputedly have the advantage of the absence of additional amino acids from affinity tags, as in the case of recombinantly expressed and purified peptides. This approach minimizes the possible interference of these tags in enzymatic assays. To further facilitate the leader peptide characterization process with regard to faster and easier chemical synthesis, linear LabA2 peptide variants with Cys → Ala mutations (LabA2*) and truncated core peptide versions (LabA2**) destined for the subsequent processing to the AB ring were used. As previously shown, such amino acid exchanges and truncations did not influence the phosphorylation or dehydration activity of LabKC. Hence, we used these peptides with leader variants to inspect the influence of leader peptide manipulations with respect to the phosphorylation and dehydration activity, i.e., initial steps in labyrinthopeptin biosynthesis.

In common peptide lantibiotic biosynthesis, the leader peptide is removed after posttranslational modifications and plays an important role in recognition by a modifying enzyme.[21] The indispensability of the leader peptide for the recognition and modification by the LabKC enzyme and consequently for the biosynthesis was demonstrated. No enzymatic processing was detected in experiments in which only the core peptide was used. Likewise, the addition of the leader peptide in trans to the reaction mixture did not result in processing activity. These findings are opposed to the experiments described for class II enzyme lacticin 481 synthetase.[23,24] In this case, the authors observed partial processing solely of the core peptide and observed an enhanced activity when incubated with leader peptide provided in trans. Furthermore, the authors concluded that the enzyme is able to recognize the core peptide alone, but the presence of the leader peptide is required for further activation, probably by shifting the equilibrium toward the active conformation.[24] With regard to our own results, reflecting the completely abolished activity for the core peptide alone and with the leader peptide provided in trans, we suggest that for labyrinthopeptin-like systems, the covalent linkage of the leader peptide with the structure peptide is essential for the processing by LabKC. Nevertheless, the possibility of enzyme activation as found for the aforementioned model system by the leader peptide binding cannot be fully excluded.

Following this finding, we attempted to pinpoint regions essential for the recognition of the leader peptide by LabKC. From experiments with peptides bearing amino acid exchanges and truncated leader variants, a minimal motif crucial for the processing could be derived. We also showed that this motif is highly conserved among sequences of other already characterized or putative lantibiotics belonging to class III.[30−35] The N-terminus of these leader peptides contains two conserved hydrophobic moieties, one acidic aspartate or glutamate followed by the highly conserved LQ motif (Figure 4). Residues more C-terminal than this motif are not crucial for the recognition as verified by Ala exchange experiments.

Subsequent examination of peptide variants with truncations in the leader peptide allowed the identification of the minimal length of the recognition site. Impairment of peptide processing was detected when a dipeptide was removed from the N-terminus, suggesting that the crucial region starts at position −18. Truncation of the C-terminal part of the leader peptide provided information about the length of the recognition motif. We were able to detect double dehydrations for the peptide variant truncated C-terminally with respect to position −9 [LabA2*$_{\Delta(-8-2)}$] and single dehydrations for the peptide truncated C-terminally with respect to position −13 [LabA2*$_{\Delta(-12-2)}$]. These findings underline the importance of the ILELQ sequence motif for the recognition by the modifying enzyme LabKC. Interestingly, it has already been suggested that a similar LFDLQ motif might be involved in the recognition by the modifying enzyme of class III lantibiotic SapB.[31] Conserved motifs within leader peptides were also identified for other classes of lantibiotics: F(N/D)LD (class I)[46] and ELXX(V/L/I)X (class II).[21]

Further investigations of the ILELQ sequence were conducted by using peptides with single and double amino acid exchanges. The I-17A mutant that was fully processed in contrast to the N-terminally truncated peptide LabA2$_{\Delta(-20-17)}$ suggests that recognition is not based on highly specific interactions at this position. This could rather be explained by the fact that the enzyme recognizes a hydrophobic patch perhaps involved in a secondary structural element, which was suggested for leader peptides from class I lantibiotics.[39] Thus, truncation experiments that involved disruption of the secondary structure would be expected to have a more significant influence on the leader peptide recognition than single substitutions. In the case of single-amino acid exchanges with Ala, the structure would be retained and the mutation of one of the three critical residues could be at least partially compensated by the two remaining hydrophobic amino acids. The possibility of secondary structure formation was also confirmed by the circular dichroism experiments of the leader peptide, showing that the LabA2 leader is able to adopt an $\alpha$-helical structure in trifluoroethanol. This is corroborated by secondary structure predictions that support a helix motif in the N-terminal part of the leader peptide containing the identified conserved ILELQ motif.

An E-15A exchange showed that the acidic side chain does not contribute to recognition as this variant was fully processed by LabKC, whereas an E-15P mutant is significantly less processed by LabKC$_{His6}$. The formation of an N-terminal helix of the leader peptide seems consistent with this E-15P mutation. The partial processing by LabKC of the E-15P

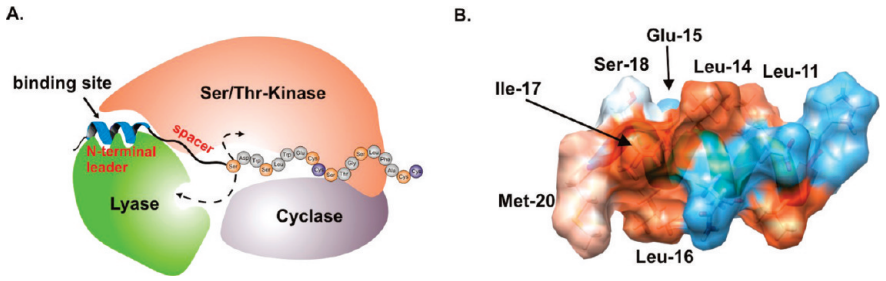


**Figure 6.** (A) Model for leader peptide-directed processing. The LabA2 core peptide is bound by the modifying enzyme LabKC via recognition of a motif in the N-terminal part of the leader peptide. The peptide remains attached to the enzyme during processing by LabKC, and a spacer determines the distance between the binding motif and the region undergoing processing, which limits the residues that can undergo modification in the active sites for phosphorylation and elimination. The model assumes the presence of one binding site for kinase and lyase domains. (B) Helical model of the N-terminal part of the LabA2 leader peptide displaying the conserved hydrophobic patch consisting of the side chains of Ile-17, Leu-16, and Leu-14.

exchange peptide could be explained by the limited ability of the enzyme to recognize the hydrophobic side chains of the conserved leader peptide motif. Our findings are corroborated by observations for class II lantibiotics where incorporation of Pro resulted in lower activity as reported for lacticin 481, which also supports the hypothesis of an $\alpha$-helix forming during binding.[24]

According to the secondary structure predictions, the suggested helix motif comprising the conserved recognition motif would be located in the N-terminal part of the leader peptide. This assumption is further strengthened by the argument that the leader peptide of SapB contains a helix-disrupting proline in the more central position (−12) that is more C-terminal than the homologous recognition motif. Hence, experiments show the chimeric SapB−LabA2 peptide is fully modified by LabKC, as seen in the expected 4-fold dehydration via LC−ESI-MS. In this context, it is interesting to note that other putative peptides from *S. erythrea* and *S. avermitilis* contain Pro residues in the corresponding part of the leader peptide, but not within the conserved N-terminus (Figure 4A). Likewise, helix-breaking glycine residues are located only in the C-terminal part of the LabA2 leader peptide. According to our findings and previous findings by other groups, the formation of an $\alpha$-helix in the leader peptide seems plausible and is in line with previous suggestions for members of classes I and II of the lantibiotics.[24,39] Detailed CD measurement studies suggested a helical structure for class I leader peptides of gallidermin, epidermin, and nisin and the class II leader of nukacin in the presence of trifluoroethanol.[39] It would be therefore interesting to evaluate if the helical conformation is induced during binding. To address these questions, further investigations need to be performed.

Arrangement of the three hydrophobic residues, I-17, L-16, and L-14, in a helical region and recognition of this hydrophobic patch by LabKC could also explain the results of replacing these residues with Asp and Arg (for I-17). For these ionic replacements, phosphorylation and dehydration activity is almost completely abolished. Thus, substitution of one of these residues cannot be compensated by the remaining two hydrophobic side chains with regard to LabKC processing. Additionally, the orientation of the glutamic side chain of E-15 on the other side of the postulated helix would explain the tolerance of acidic residues at this position by LabKC as recognition would rather be accomplished by the hydrophobic side of the helix (Figure 6). However, all three positions (I-17, L-16, and L-14) seem to be critical for recognition. Introduction of Asp at any of these positions completely abolishes peptide processing, probably because of electrostatic effects. The suppression of dehydration activity by incorporation of charged residues at conserved hydrophobic positions is also consistent with similar results for class II lantibiotics, i.e., mutacin II, nukacin, and lacticin 481, obtained independently from in vivo and in vitro studies.[24,41,42]

On the other hand, the loss of one of the crucial residues mentioned above via a replacement with Ala could be compensated by the remaining two hydrophobic side chains of Leu or Ile as observed by processing of corresponding peptide substrates by LabKC. The Ala exchanges further showed that positions L-16 and L-14 are more critical as in these cases significant accumulation of phosphorylated intermediates was observed. Surprisingly, one of the remaining substrate variants, peptide I-17N, was not processed by LabKC, although the Thr exchange at this position (I-17T) with a similar polarity was very well tolerated. Exchanges of I-17, L-16, and L-14 with other nonpolar amino acids (I-17F, I-17L, L-16V, and L-14V) identified in homologous sequences did not diminish dehydration activity substantially; however, minor differences between individual peptide variants could be observed (Table S1 of the Supporting Information). The double Ala exchanges in the recognition motif of the leader peptide result in complete or almost complete loss of phosphorylation and dehydration activity (Figure 5). The importance of the polar residue Q-13 was underlined by investigating two double mutants (LabA2**$_{\text{I-17A,Q-13A}}$ and LabA2**$_{\text{L-14A,Q-13A}}$) in which a single Ala substitution of Q-13 was tolerated well but in conjunction with concomitant I-17 → A or L-14 → A substitutions led to an almost complete loss of processing by LabKC. Interestingly, the level of processing of the LabA2**$_{\text{L-14A,Q-13A}}$ mutant was significantly lower (Table S1 of the Supporting Information) than that of LabA2**$_{\text{I-17A,Q-13A}}$, which suggests that I-17 is comparatively less important for recognition. This finding is in agreement with single Ala substitutions. Remarkably, the loss of processing activity by substitutions of hydrophobic amino acids Leu and Ile (LabA2**$_{\text{I-17A,L-16A}}$ and LabA2**$_{\text{L-16A,L-14A}}$) with the hydrophobic amino acid Ala points to more demanding sterical requirements for recognition by LabKC. Moreover, the LabA2**$_{\text{L-16A,L-14A}}$ mutant suggests that enzyme recognition is not limited to only one face of the proposed helix, because both Leu residues would be located apart from each other in this conformation.


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Additional confirmation of the importance of the proposed motif was realized in the experiment in which the ILELQNL motif was shifted C-terminally to the middle of the leader peptide. Experiments with truncated peptides LabA2*$_{\Delta(-8-2)}$ and LabA2*$_{\Delta(-12-2)}$ highlighted a crucial distance dependency within the precursor peptide for serine dehydration. In these cases, dehydration events occurred only in the C-terminal part of the core peptide, suggesting the existence of a spacer region between the recognition site of the leader peptide and the core peptide. We propose that the main function of this spacer region is to maintain proper distance requirements. This suggestion is also supported by the analysis of leader peptides from other class III lantibiotics (Figure 4A), whereas a higher sequential diversity is observed in contrast to the conserved recognition motif. Similar observations for distance dependence between the leader peptide and the processing sites were made for the class II lantibiotic lacticin 481.[45] In this case, however, the C-terminal part of the leader peptide is more important for the binding as shown by truncation experiments.

Our findings elaborated for the leader peptide of labyrinthopeptins and also the leader of SapB are reminiscent of those for the well-characterized leader peptide of microcin B17.[28,40,43,44] In this case, both features, the proposed recognition site represented by the hydrophobic patch and the function of the C-terminal part of the leader peptide as a spacer region between the recognition site and processing sites, are remarkably consistent with our findings. It is interesting to note that for microcin B17 a helical structure in the leader peptide was observed by NMR analytics in the presence of trifluoroethanol.[40] This postulated helical structure located in the N-terminus of the leader peptide is followed by a polyglycine linker, having the only function of limiting the residues that can be processed in the active side for the cyclodehydration reaction. It is remarkable that very similar modes of recognition of the leader peptide evolved independently for two very different posttranslational modification machineries of microcin B17 and labyrinthopeptin. Very recently, a detailed analysis of the leader peptide of class I lantibiotic nisin led to a similar model with the FNLD motif responsible for the recognition located in the N-terminal part of the leader peptide.[46] In addition, similar distance requirements for the processing site with respect to the leader peptide were observed.

**Model for Enzymatic Processing.** In a proposed model for leader peptide recognition and modification of the precursor peptide, the leader peptide would be recognized and bound by LabKC via the hydrophobic patch represented by the ILELQ motif in the N-terminal region forming an $\alpha$-helical structure (Figure 6). During the processing, the core peptide would be delivered to the active sites of the subdomains of LabKC via a spacer region providing necessary distance requirements. One phosphorylation event would be directly followed by the elimination of the phosphate group as no multiply phosphorylated intermediates are observed during processing at high substrate concentrations. This observation might be explained by the high binding affinity of the phosphate moiety in the active site of the lyase domain.[14] Subsequent to the dehydration of the precursor peptide, cyclization, i.e., formation of labionin, would occur, as well as formation of the cystine of ring C. At this point, the mechanism of cleavage of the leader peptide is still unknown. There are no conserved motifs for protease recognition as determined for class I and II lantibiotics.[1,6] Also, there is neither a protease encoded in the *lab* gene cluster nor a unifying sequence motif for a protease apparent from sequence homologies to other class III lantibiotics.

In these studies, we showed the importance of the leader peptide for the processing of the precursor peptide by the modifying enzyme LabKC with respect to phosphorylation and dehydration activities. We did not, however, investigate the influence of the leader peptide on the cyclization reaction, which is crucial for improving our understanding of the mechanistic aspects of the overall precursor peptide processing. It has to be noted that the proposed recognition function of the conserved motif could not be fully investigated. Because of difficulties with the binding experiments, we could not clearly demonstrate if the lack of processing for some of the tested peptides was due to the binding event. We cannot exclude the possibility that peptides that were not processed were still bound by the enzyme but more subtle effects (e.g., proper positioning) impaired the processing. We will address these issues in future studies. The proposed model for the recognition will be also evaluated by further NMR conformational studies of the leader peptide.

## SUMMARY

In summary, the results presented in this study give the first insights into the sequence requirements of the leader peptide for recognition of class III lantibiotics by their processing enzymes. Using the example of the leader peptide of labyrinthopeptin A2, it was demonstrated that three specific hydrophobic residues in an N-terminally conserved motif are crucial for the processing. A reduced dehydration activity was observed when exchanging these residues against Ala, and conversion was completely abolished by exchange with charged amino acids. Furthermore, this conserved motif was additionally mapped with truncation experiments revealing the minimum length of the leader required for the processing and identifying the conserved N-terminal part as the recognition site necessary for proper processing. The C-terminal part of the LabA2 leader peptide plays only the role of a spacer delivering the core peptide to the active sites of the kinase and lyase domains of LabKC.

## ASSOCIATED CONTENT

### Supporting Information

Detailed information about chemical peptide synthesis and further mass spectra from the LabKC assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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